Clinical manifestations and characterization of extra-intestinal *Vibrio cholerae* non-O1, non-O139 infections in Denmark

A. Dalsgaard[1,*], *A. Forslund*[1], *A. Hesselbjerg*[2] *and B. Bruun*[2]

[1]Department of Veterinary Microbiology, Stigböjlen 4, The Royal Veterinary and Agricultural University, DK-1870 Frederiksberg C, Denmark, [2]Department of Clinical Microbiology, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark

*Tel: +45 35 282720 Fax: +45 35 282757 E-mail: ad@kvl.dk

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Vibrio cholerae strains of serotypes O1 and O139 are the only causes of cholera. However, other serotypes of *V. cholerae*, so-called non-O1, non-O139 serotypes, are increasingly being associated with human disease, in particular diarrhoea [1–5]. Non-O1, non-O139 strains of *V. cholerae* have also been isolated from blood, from wound and ear infections and from other clinical sites; such infections comprise up to 50% of the reported non-O1, non-O139 infections in the USA [6]. Extra-intestinal invasive infections have also been described from southern Europe [7,8]. Isolation of *V. cholerae* non-O1, non-O139 from wound and ear infections have with few exceptions all been associated with water exposure, mainly to seawater [6,9]. However, little is known about the occurence and characteristics of *V. cholerae* non-O1, non-O139 infections in temperate areas in Europe, particularly northern Europe. Cases of non-O1, non-O139 gastrointestinal infections in the USA seem to occur in the late summer and early fall when water temperatures and the numbers of non-O1, non-O139 strains are high in the aquatic environment (water and oysters) [10]. A similar positive correlation between water temperatures and the incidence of extra-intestinal non-O1, non-O139 *V. cholerae* infections would be expected. In the present study, we report the clinical manifestations and strain characteristics of eight extra-intestinal *V. cholerae* non-O1, non-O139 infections acquired between 1994 and 1998 in Denmark.

The clinical strains examined are listed in Table 1. The strains were obtained from cases of *V. cholerae* non-O1, non-O139 infections which were sent to Statens Serum Institut in Copenhagen for final identification or verification. Strains were identified as *V. cholerae* using criteria described in the *Manual of Clinical Microbiology* [11] and were tested for agglutination in polyvalent *V. cholerae* O1 antiserum (Difco, Detroit, MI, USA). Each strain was further examined serologically by Dr T. Shimada at the National Institute of Infectious Diseases, Tokyo, by the slide agglutination test and designated according to an extended serotyping system (O1 to O140) established by Shimada et al. [12]. Strains were examined by the colony hybridization technique for DNA sequences encoding a heat-stable enterotoxin (NAG-ST) and cholera toxin (CT) using alkaline phosphatase-labeled oligonucleotide probes consisting of 16 and 23 base pairs, respectively [13–15]. NAG-ST and CT are well recognized virulence genes among *V. cho-*

Table 1 Summary of clinical data of eight patients from whom extra-intestinal *Vibrio cholerae* non-O1, non-O139 was isolated in Denmark between 1994 and 1998

Case no.	Date of exposure	Age/sex	Exposure to water	Clinical presentation	Pure culture	O-serotype[a]
1	July 1994	82/F	Unknown	Septicemia	Yes	–[e]
2	July 1994	65/M	Seawater	Ulcus cruris	Unknown	O10
3	August 1994	14/M	Seawater	Otitis media acuta	No[b]	O19
4	August 1994	42/M	Seawater	Otitis media chronica	Yes	O52
5	August 1994	10/M	Seawater	Otitis media serosa, tubulation	Yes	O10
6	April 1996	10/M	Fresh water/swimming pool	Otitis externa	No[c]	O15
7	September 1997	13/M	Fresh water	Otitis externa, cholesteatoma	Yes	O26
8	March 1998	36/M	Unknown	Otitis externa	No[d]	O19

[a]O-serotype according to the extended serotyping scheme by Shimada et al. [12]. [b]*Shewanella putrefaciens*/ algae also isolated from ear. [c]*Staphylococcus aureus* also isolated from ear. [d]*Pseudomonas* spp. also isolated from ear. [e]Strain not available.

lerae non-O1, non-O139 strains associated with gastrointestinal diseases [16].

Susceptibility of the *V. cholerae* isolates to penicillin, ampicillin, cefuroxime, ceftriaxone, gentamicin, ciprofloxacin, tetracycline and chloramphenicol were determined by the Epsilon test (E test) method (AB Biodisk, Sweden) according to the manufacturer's instructions. Tablet diffusion was done with Neo-Sensitabs (Rosco, Taastrup, Denmark) containing trimethoprim-sulfamethoxazole, polymyxin E and the vibriostatic agent O/129 (150 μg). Repeated plasmid analyses were done using appropriate controls as described previously [17,18].

PFGE typing using the restriction enzyme *Not*I was carried out by using a modified contour-clamped homogeneous electric field (CHEF) system (Pulsaphor Plus, Pharmacia LKB, Sweden) as described by Dalsgaard et al. [19].

A summary of important clinical data of the eight cases of extra-intestinal cases of *V. cholerae* non-O1, non-O139 is presented in Table 1. The first patient (case no. 1) was a previously healthy woman who presented with primary bacteremia without a known bacteriological or clinical focus, including no preceding diarrhea. Cultures of stool samples were repeatedly negative for *V. cholerae*. The patient was treated with ampicillin and gentamicin with good effect. This strain of *V. cholerae* was unfortunately lost and thus not available for further characterization. The severity of the leg ulcer of the 65-year-old male (case no. 2) necessitated debridement and skin transplantation. The remaining six cases all involved ear infections, of which three (case nos 4, 5 and 7) were acute exacerbations on the basis of chronic conditions, involving tubulation and cholesteatoma. Of the remaining three episodes, one patient (case no. 3) had never had previous ear problems, another (case no. 6) had been tubulated as a young child, but had been without ear problems for the last 4 years, and there was no history on the last patient (case no. 8). In the patient presenting as acute otitis media (case no. 3), paracentesis was performed. The other five ear-patients were treated with hydrocortisone–polymyxin–tetracycline ear drops with apparent good effect. In one case (case no. 7), however, a cholesteatoma was diagnosed, necessitating debridement and tympanoplasty.

The seven clinical strains available for characterization showed the typical biochemical characteristics of *V. cholerae*, including the ability to produce lysine decarboxylase, a positive ONPG test, sensivitity to O/129 and growth in a medium without NaCl [11]. None of the strains agglutinated with the polyvalent O1 antiserum. Further serotyping revealed that two strains belonged to serotypes O10 and O19 each, while single strains belonged to serotypes O15, O26 and O52 (Table 1). The cases yielding identical serotypes did not appear to be epidemiologically related. None of the seven strains contained plasmids in repeated testing, nor did they contain NAG-ST and CT genes, as shown in the colony hybridization studies.

Genotyping of the seven strains by PFGE revealed very different types differing from each other by more than seven fragments, showing that the strains were not genotypically related (results not shown).

Overall, little difference was found among the *V. cholerae* examined in their susceptibilities towards any of the antibiotics tested. According to the NCCLS performance standards, minimum inhibitory concentration (MIC) values, which are shown in brackets, all seven strains were found by the E test method to be susceptible to ampicillin ($\leq$ 8 mg/L), cefuroxime ($\leq$ 8 mg/L), ceftriaxone ($\leq$ 8 mg/L), gentamicin ($\leq$ 4 mg/L), ciprofloxacin ($\leq$ 1 mg/L), tetracycline ($\leq$ 4 mg/L) and chloramphenicol ($\leq$ 8 mg/L) , but resistant to penicillin ($\leq$ 0.12 mg/L) [20]. Similar results were obtained by the tablet diffusion test, which also demonstrated susceptibility to O/129 and trimethoprim–sulfamethoxazole and resistance to polymyxin E with the exception of one strain.

This study of *V. cholerae* non-O1, non-O139 isolated in Denmark between 1994 and 1998 presents clinical and bacteriological characterization of extra-intestinal *V. cholerae* infections acquired in Scandinavia. To the best of our knowledge, these eight patients comprise all of the known cases of extra-intestinal *V. cholerae* infections in Denmark during this period. Previous studies have mainly described cases of extra-intestinal infections from subtropical and tropical areas, in particular in the USA [9]. In the present study, *V. cholerae* non-O1, non-O139 was isolated in pure culture from four patients, whereas specimens from the cases of three patients gave growth of other external otitis pathogens (Table 1). Thus, our findings suggest that the isolated *V. cholerae* strains were associated with the clinical manifestations presented. However, it should be noted that virulence factors enabling *V. cholerae* to cause extra-intestinal infections have not been demonstrated to date. Our finding that none of the strains contained the enterotoxin genes NAG-ST and CT suggest, as expected, that these genes are not involved in extra-intestinal infections.

Five patients, of whom four had a history of exposure to seawater, obtained their infections in July and August 1994. It has previously been reported that during this summer, which was the hottest recorded at the Danish Meteorological Institute with bathing water temperatures above 20 °C for several weeks, 11 patients acquired wound infections with *V. vulnificus* [21]. A later environmental study of *Vibro vulnificus* in Danish costal waters showed a positive correlation between the water temperature and the numbers of *V. vulnificus* [22]. These findings are corroborated by an earlier Danish report of an increase in the number of extra-intestinal infections caused by *V. parahaemolyticus* and *V. alginolyticus* during warm summers from 1987 to 1992 [23]. As the ecology of *V. cholerae* non-O1, non-O139 resembles that of other human pathogenic vibrios in several aspects, it is likely that relatively higher numbers of non-O1, non-O139 in Danish coastal waters together with an

increased bathing activty in 1994 were associated with the isolation of *V. cholerae* from patients, mainly with ear infections. In contrast to other halophilic human pathogenic *Vibrio* spp., *V. cholerae* can survive and multiply in fresh water. Thus, extra-intestinal infections with *V. cholerae* may occur in patients exposed to fresh water, as was illustrated by the isolation of *V. cholerae* from two patients with histories of exposure apparently only to fresh water. One of these cases had repeated exposure to water in swimming pools prior to the development of otitis externa.

The incidence of *V. cholerae* non-O1, non-O139 infections in the study period was most probably higher than reported, since less severe cases of ear and wound infections presumably would not have been presented to physicians. Although ear and wound specimens are not cultured on media specific for *Vibrio* spp. (thiosulfate–citrate–bile salt–sucrose (TCBS) agar) in clinical microbiology laboratories in Denmark, *Vibrio* species are expected to be recovered, as they readily grow on blood agar plates routinely used by the laboratories. Our study shows that physicians in temperate countries should be aware of the possibility of *V. cholerae* non-O1, non-O139 extra-intestinal infections, especially in patients seen during warm summers when water temperatures and recreational activities in water are high.

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